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Morphological study of human embryos co-culturing in a time-lapse incubator

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ABSTRACT

In the present study, a co-culturing method is proposed, which consists in culturing two embryos in the same plate. The aim of this study was to analyze the impact of embryo co-culturing on the development and quality of human embryos, with the final goal to perform the Intra- cytoplasmic sperm injection procedure. An improvement of the embryo development and implantation rates was expected to achieve in those co-cultured embryos.

In order to analyze the co-culturing impact in the embryo features, morphological factors are evaluated in several embryo developmental stages. Monitoring these changing morphological events is currently feasible thanks to advanced strategies such as the time-lapse incubator.

After the ovarian treatment, which included superovulation, ovarian puncture and removal of cumulus-corona, and the sperm selection, the injection procedure was carried out in a total of 297 oocytes from 21 patients. Then, morphological changes of the embryos were supervised and analyzed before proceeding to cryopreserve them. In this way, the transference of the best quality embryo could be performed and so, enhance the gestation and birth rates.

During the embryo development, a zygot scoring was applied to select those fertilized eggs with 2 pronuclei (2PN) and to discard the non-fertilized and degenerated oocytes, as well as those with abnormal fertilization. The fertilization rate of co-cultured embryos was higher than non-co-cultured embryos but non-statistically significant (85.05% and 78.64% respectively, $P>0.05$). No significant differences were shown neither at day 3 morphological evaluation nor embryo compaction at day 4 ($P>0.05$). Significantly ($P<0.05$) higher proportions of blastocysts at day 5 were obtained in the co-cultured embryos group, whereas at day 6 and day 7 blastocysts rates weren't statistically different ($P>0.05$). For implantation rate, the hCG blood test and the analysis of the evolutive implantation weren't significantly different ($P>0.05$), however the clinical implantation had significant differences ($P<0.05$) among co-cultured and non-co- cultured embryos group.

It may be concluded that the co-culturing influenced beneficially the blastocyst percentages at day 5, as well as an improvement in the implantation rate. Hence, this new method might become a potential tool to improve the overall embryo development and so, benefit further assisted reproductive techniques.

RESUM

En aquest assaig es proposa un sistema de co-cultiu mitjançant el cultiu de dos embrions en una mateixa placa. L'objectiu principal d'aquest estudi va ser determinar l'efecte del co-cultiu d'embrions en el desenvolupament i la qualitat dels embrions humans mitjançant la injecció intracitoplasmàtica d'espermatozoides. S'espera observar millores en el desenvolupament embrionari i en les taxes d'implantació en els embrions que es troben co-cultivats.

Per tal d'analitzar l'impacte del co-cultiu en les característiques de l'embrió, s'avaluen els factors morfològics en diferents etapes del desenvolupament de l'embrió. L'anàlisi d'aquests canvis morfològics es realitza mitjançant un incubador time-lapse que permet fer un seguiment continu del desenvolupament embrionari.

Un cop realitzat els diferents tractaments dels òvuls, com la superovulació, la punció ovàrica i l'eliminació de les cèl·lules del cúmulus-corona, i la selecció espermàtica, es duu a terme la injecció ovàrica en 297 oòcits provinents de 21 pacients. Durant el cultiu, es va efectuar un seguiment complet de l'embrió per tal de fer una avaluació dels paràmetres morfològics. Després de la criopreservació, l'embrió de millor qualitat va ser transferit.

Per tal de dur a terme una revisió del desenvolupament embrionari, es va realitzar una classificació dels zigots seleccionant aquells fecundats que presentaven 2 pronuclis (2PN) i descartant els embrions no fecundats i degenerats, com també aquells amb fecundació anòmala. La taxa de fecundació dels embrions co-cultivats va ser més alta que en els embrions no co-cultivats, tot i això no es van observar diferències significatives (85.05% i 78.64% respectivament, $P>0.05$). En l'anàlisi morfològica a dia 3 i en la compactació de l'embrió a dia 4 no es van constatar diferències significatives ($P>0.05$). Pel que fa a la taxa d'implantació, el test d'hCG en sang i la implantació evolutiva no van presentar diferències significatives ($P>0.05$), tot i això en la implantació clínica es van trobar diferències significatives ($P<0.05$) entre els dos grups d'embrions co-cultivats i no co-cultivats.

Es pot concloure que el co-cultiu ha influït positivament en els percentatges a dia 5 dels blastocists, com també ha millorat la taxa d'implantació. Així doncs es pot concloure que aquesta nova tècnica de co-cultiu podria convertir-se amb una eina de gran potencial ja que pot millorar el desenvolupament embrionari i conferir avantatges a altres tècniques de reproducció assistida.

RESUMEN

En este ensayo se propone un sistema de co-cultivo mediante el cultivo de dos embriones en una misma placa. El objetivo principal de este estudio fue determinar el efecto del co-cultivo de embriones en el desarrollo y la calidad de los embriones humanos mediante la inyección intracitoplasmática de espermatozoides. Se pretende observar mejoras en el desarrollo embrionario y en las tasas de implantación en los embriones que se han co-cultivado.

Con el fin de analizar el impacto del co-cultivo en las características del embrión, se evalúan los factores morfológicos en diferentes etapas del desarrollo del embrión. El análisis de estos cambios morfológicos se realiza mediante una incubadora time-lapse que permite realizar un seguimiento continuado del desarrollo embrionario.

Tras realizar los distintos tratamientos de óvulos, como la superovulación, la punción ovárica y la eliminación de las células del cúmulus-corona, y la selección espermática, se lleva a cabo la inyección ovárica en 297 oocitos provenientes de 21 pacientes. Durante el cultivo se efectúa un seguimiento completo del embrión con el fin de realizar una evaluación de los parámetros morfológicos. Tras la criopreservación, el embrión de mejor calidad fue transferido.

A fin de realizar una revisión del desarrollo embrionario, se clasificaron los zigotos seleccionando aquellos fecundados que presentaban 2 pronúcleos (2PN) y descartando los embriones no fecundados y degenerados, así como aquellos que presentaban una fecundación anómala. La tasa de fecundación de los embriones co-cultivados fue más alta que en la de los embriones no co-cultivados, sin embargo no se observaron diferencias significativas (85.05% i 78.64% respectivamente, $P>0.05$). En los análisis morfológicos a día 3 y en la compactación del embrión a día 4 no se constataron diferencias significativas ($P>0.05$). Respecto a la tasa de implantación, el test de hCG en sangre y la implantación evolutiva no presentaron diferencias significativas ($P>0.05$), aunque sí se observaron diferencias significativas en la implantación clínica ($P<0.05$) entre los dos grupos de embriones co-cultivados y no co-cultivados.

Se puede concluir que el co-cultivo ha influido positivamente en los porcentajes a día 5 de los blastocitos, mejorando la tasa de implantación. Así, podemos concluir que el co-cultivo de embriones es una herramienta de gran potencial que podría beneficiar tanto el desarrollo de los embriones como posteriores técnicas de reproducción asistida.

I. INTRODUCTION

Infertility is a source of social and psychological suffering for both men and women and can place great pressure on the relationship within the couple. One in six couples of any society remains infertile and 10% of them need help of assisted reproductive technology (ART) (Mosammat, 2010). ART refers to all technology and procedures were gametes are manipulated outside the body for the purpose of establishing a pregnancy. These procedures entail the *in vitro* handling of both human oocytes and sperm, and/or embryos. Briefly, several techniques are included such as in vitro fertilization and embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy (Zegers-Hochschild et al., 2009). Unfortunately, there still remain a great number of barriers for these treatments to date like high cost of treatment, poor result, social stigma and superstitious beliefs which are hindering the extent of ARTs among the community.

1. INTRACYTOPLASMIC SPERM INJECTION (ICSI)

Assisted reproduction techniques, in particular in-vitro fertilization and intracytoplasmic sperm injection, are the most advanced forms of infertility treatment. Specifically, the Intracytoplasmic Sperm Injection (ICSI), has gained an increasing interest in the last decades among the medical community because of its success rates (Palermo *et al.*, 1993).

Briefly, this technique consists in a micro-manipulation process where a single spermatozoid is injected directly inside the oocyte *ex vivo* (Figure 1). Numerous studies have proved that following this technique, and improved quality and implantation of the embryo was reached, along with early divisions during the first stages of the embryonic development, which has been stated as biological indicator of the embryo potential (Lundin et al., 2001; Giorgetti et al., 2007; Lemmen et al., 2008).

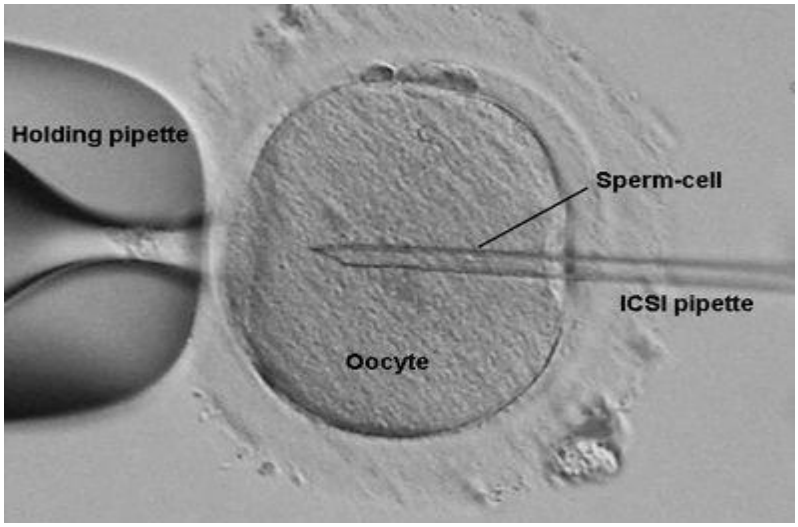

Figure 1: Microscopic image showing the Intracytoplasmic Sperm Injection procedure. Source: http://www.trianglen.com/ivficsi---step-by-step.html

Notwithstanding this cautious optimism, further efforts are still required in order to improve the mentioned medical procedure so as to obtain enhanced results in terms of survival, fertilization and implantation of the injected embryos as well as to increase the embryo quality.

2. MONITORING OF EMBRYONIC DEVELOPMENT

Once the fecundation has occurred, the embryonic development has been traditionally monitored *in vitro* at selected time points; however, this process entails a major backward as the environment where the embryo is growing is profoundly disturbed by the manipulator and it can interfere with its proper developing.

With the emergence of the time-lapse technology, where a real-time monitoring of the embryo is conducted (Payne et al., 1997), this previous problem has been overcome. The time-lapse technology enables to monitor in real-time the embryonic development uninterruptedly so a better comprehension of the morphological events taking place can be obtained as well as the crucial points in their maturation. Moreover, a broader understanding of the morphokinetics can be used as a predictor of embryo implantation and may further improve the selection of viable embryos (Messeguer et al., 2011).

Summarizing, this technology enables to properly identify the key points in the early stages of the embryo development, contributing to improve the ARTs (Mio, 2006) as well as the implantation rates by a better understanding of the embryos dynamic (Aparicio et al., 2013). Also, the accuracy in embryos scoring may benefit due to the possibility of review the embryo development images an unlimited number of times (Lemmen et al., 2008).

3. CO-CULTURE OF EMBRYOS

In the last years, numerous studies have evidenced that the culture conditions in which the embryo develops do have a notorious impact on the implementation rate (Rizos et al., 2008). For this reason, researchers have extensively focused on trying to mimic the in vivo conditions in order to improve the quality of cultured blastocyst and so, enhance the rates of success in d assisted reproductive techniques.

Co-culture has been postulated as a potential tool which can favour the production of high quality blastocyst. Briefly, co-culture is defined as the culture of embryos along with a specific somatic cell line in order to simulate the physiological conditions and facilitate the embryo development (Figure 2). These cells, generally referred as “helpers”, when cultured with the embryos can contribute to a successful implantation. In particular, these strategy might be notably beneficial for patients suffering recurrent implantation failures or when working with poor quality embryos (Menlick et al, 2016). Briefly, the main goal of embryo co-culture is to counteract the negative effect of deleterious components and their oxidative action that may interfere in the proper development of the embryo in its early stages. Additionally, cells constituting the co-culture can release advantageous factors for the embryo (Joo et al., 2001).

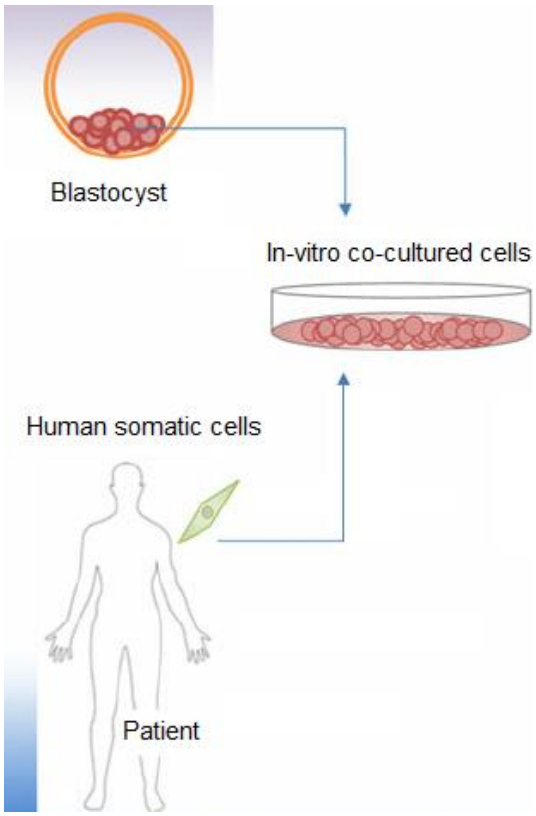

Figure 2: Schematic diagram of the co-cultured method. Self-source.

As mentioned herein, the basic elements required to establish a co-culture are the embryo and somatic cells; nevertheless, some requirements need to be met as only autologous cells can be used. Precisely, cells from an animal origin cannot be elected as it supposes a great risk of infection (Melnick et al., 2016).

Co-culture for reproductive goals was first introduced in animals (Cole et al., 1965) and given the great success achieved in different studies conducted in numerous years (Camous et al., 1984; Gandolfi et al., 1987); this procedure was finally implemented for human use. Here, numerous benefits could be appreciated in the embryo morphology and the implantation rate as well as the embryo quality (Wiemer et al., 1989; Bongso et al., 1989; Feng et al., 1996). It has been stated that epithelial cell possess the broadest potential when working with co-cultures, in high contrast with fibroblast which did not provide any advantageous effect when embryonic development was studied. In a similar fashion, other cells lines such as fallopian tube cells have also been cultured successfully (Ouhibi et al., 1989) along with other established cell lines (Ménézo et al.,1990)

Despite the great number of studies published referring to the use of somatic cell lines for co-culture of embryos, to date not a single publication has surfaced proposing an alternative use of germinal cells for this goal. For this reason, in this present work we have focused on the co-culture of two embryos belonging to a same patient in order to evaluate the effect of the own embryo in the quality and implantation rate of its homologous and vice versa.

II. OBJECTIVES

The main objective of the present work is to assess the impact of co-culturing two embryos belonging to same patient in a single dish on the development and final quality of human embryos obtained after ICSI procedure.

In order to accomplish the present objective, the following work plan is proposed:

1. To analyze different morphological parameters of the embryos and their further categorization in terms of embryo quality following standardized guidelines. To reach this objective, images from the embryos retrieved in real-time from an incubator will be evaluated.

2. To contrast factors such as embryo fertilization rate, embryo-to-blastocyst rate, cryopreservation rate and implantation rate between co-cultured and non-co-cultured embryos.

3. To assess whether an improvement in the implantation rate after a co-cultured incubation occurred in comparison with a non-co-cultured incubation.

The hypothesis formulated for this present work is that co-culturing embryos in a single dish leads to a better embryo development and a higher implantation rate of the embryos as it has been observed in recent investigations developed in the facilities where the work will be conducted.

III. METHODOLOGY

1. EXPERIMENTAL DESIGN

The current essay was conducted with a total number of 21 patients. A preliminary screening of the patients was performed so only patients with co-cultured and non-co-cultured embryos participated whilst patients with embryos exhibiting chromosomal abnormality or negative pre-implantation genetic diagnostic were excluded. In parallel, data like patient age, or receptor and donor age was also considered. An identifier number was assigned to each one of the embryos of every patient. Specifically, co-cultured embryos were from now on designated as 2 per drop, whereas the single embryos were designated as 1 per drop. Numbers were allocated in order to localize the embryos in their corresponding well. An age threshold was applied in some analyses. The total number of patients were grouped into patients younger than 35 years old (<35) and patients equal or greater than 35 years old (≥35).

2. SUPEROVULATION AND OVARIAN PUNCTURE

Acquiring a large number of oocytes is an important requirement so as to further apply the pertinent assisted reproductive techniques. To this end, the superovulation technique was applied, which consist of an ovarian stimulation using exogenous gonadotropins so patients undergoing an ICSI cycle might increase their number of oocytes collected (Guzick et al., 1999). After stimulation, oocytes were removed using the ultrasound-guided oocyte retrieval (Wickland et al., 1985), an assisted reproductive technology (ART) performed vaginally whereby oocytes are retrieved by aspirating the follicular fluid from the antral follicles (Royal College of Nursing, 2004).

3. REMOVAL OF CUMULUS-CORONA (CC) CELLS

CC cells surrounding the oocyte must be removed prior to injection procedure. To this end, several treatments were applied to the oocytes. Firstly, plates containing fertilization medium (Vitrolife G-IVF) were covered with Vitrolife Ovoil and incubated at 37ºC and 6% CO_2 24 hours before their utilization. Retrieved oocytes were then cultured in the fertilization medium which simulates *in vivo* conditions during one hour in order to equilibrate them, and a first cleansing was carried out so as to get rid of excess tissue and other impurities. Subsequently, oocyte denudation was performed with Hyase 10X Vitrolife, a physiological salt buffer containing hyaluronidase and human serum albumin. The hyaluronidase triggers an enzymatic reaction that removes the surrounding cells from de oocyte (Van de Velde et

al., 1998). This procedure was complemented with mechanical denudation using a pipette (Gianaroli et al., 2000). Extremely accurate pipetting needs to be performed so as to prevent oocyte damages. The cleaning procedure was repeated several times so as to ensure a complete denudation and the remaining oocytes were cultured in G-MOPS Vitrolife medium. Finally, prior to the injection procedure, a screening of the oocytes was performed to classify them as only metaphase II (MII) and metaphase I (MI) oocytes are used in further procedures.

4. SPERM SELECTION

A fresh sample of semen was collected as described in the ESHRE guideline (Gianaroli et al., 2000) at the same time that the ovarian puncture was performed. Before its manipulation, the semen was allowed to rest around 30 minutes so as to naturally liquefy. Briefly, the spermatozoa selection was performed using a Fertile Microfluidic Sperm Sorting Chips. This microfluidic technology (Sia et al., 2003) permit isolating the most motile spermatozoa from those ones compromised. Interestingly, the selected spermatozoa have a higher DNA integrity (Schulte et al., 2007), avoiding the sperm-damaging procedures associated with swim-up and gradient centrifugation (Mahadevan et al., 1984).

5. INJECTION PROCEDURE

Intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992) was carried out as described by Joris et al. (1998). This ART consists in a micro-manipulation process where a single spermatozoon is injected directly inside the oocyte by means of subjection and holding pipettes. This procedure is indicated for masculine and non-diagnosed infertility. An inverted microscope with a microinjector was used to perform the injection procedure. During all process, oocytes were preserved in G-MOPS Vitrolife medium. MI oocytes require a previous maturation treatment *in vitro* before proceeding to their injection (De Vos et al., 1999).

6. EMBRYO CULTURE

The fertilized oocytes were cultured in a Geri dish with SAGE 1-step medium for a week in a Geri incubator. The Geri technology allows professionals to monitor the process of development of the embryo without disturbing the incubating conditions in high contrast with other equipments, where oocytes are required to be removed from the incubator in order to supervise them. Here, eleven photographs were taken every 5 minutes in the z axis in real-time so as to control the embryos. Generally, embryos were cultured until the blastocyst stage was reached, which approximately occurred around day 5 (D5) or day 6 (D6) although some of them with a slower rate of development were kept in the incubator until day 7 (D7).

At this point, a scoring of the embryos was carefully conducted to select those of a better quality according to the ASEBIR consensus as this will determine the implantation rates.

6. CRYOPRESERVATION AND EMBRYO TRANSFER

The selected blastocysts with the best quality were cryopreserved with a vitrification technique (Fahy et al., 1984). Embryo vitrification was mainly performed so as to allow the transference of embryos in the next ovarian cycle; thus, the patient could recover from the superovulation and the uterus would be more propitious to the embryo implantation (Gianaroli et al., 2000). A single blastocyst was transferred to the patient uterus following the guidelines (Practice Committee of the American Society for Reproductive Medicine, 2017).

7. ZYGOTE SCORING

In order to classify zygotes, the presence of pronuclei was studied in the images obtained from the Geri incubator, which generally tends to occur from 16 to 19 hours post ICSI (Nagy et al., 1994; Boada and Ponsà, 2008). Following ASEBIR consensus, zygotes with one polar body were discarded, regardless of pronuclei number. Two polar body zygotes with one pronucleus were also discarded due to its susceptibility to present aneuploidies (Mateo et al., 2013) and parthenogenic activation (Staessen and Van Steirteghem, 1997). Zygotes presenting three pronuclei were also rejected as they come from an abnormal fertilization and prone to suffer a triploidy (Palermo et al., 1995). The absence of pronuclei is equivalent to a non-fertilized oocyte so they were neither considered, as well as degenerated embryos that present lysis of the oocyte (Rosen et al., 2006). Contrastingly, zygotes with two polar bodies and two pronuclei were account as fertilized oocytes and were selected to perform the morphological analyses.

8. EMBRYON IMPLANTATION

In order to confirm the positive implantation the embryo different tests were required. On the one hand, a blood beta-hCG test was performed to determine if the embryo had adhered to the uterus wall. In physiologic conditions, the levels of human chorionic gonadotropin hCG in circulating blood are minimum but when pregnancy is produced, these hCG levels increase significantly to maintain the corpus luteum and progesterone production during the first trimester (Navot et al., 1992). After a beta-hCG confirmation, the clinical pregnancy was checked, which is defined as the presence of heartbeats 5 weeks after embryo transfer (Hardarson et al., 2008). Finally, evolutive pregnancy is confirmed when the gestation develops correctly and the placenta and hormone levels follow their normal course.

9. MORPHOLOGICAL CLASSIFICATION

Similarly, defining features of the embryos were evaluated using the images retrieved from the Geri incubator at different time points. Following the established consensus of ESHRE (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011), at 72 hours (day 3; D3) post-ICSI it was assessed the number of cells per embryo was assessed along with an analysis of the cellular symmetry of the embryos and their fragmentation degree. Based on these results, a further morphological classification of the embryos was performed following the ASEBIR guideline, although some variations were applied as gathered in Table 1. On the fourth day (D4) of the embrionary development, the degree of morula compaction was analyzed. Finally, blastocysts were evaluated according to blastocoel expansion, inner cellular mass and trofectoderm, classifying at D5 according to the classification proposed by Gardner (Gardner et al., 1999).

Table 1. Embryos classification at day 3 according to morphological parameters. Source: ASEBIR, 2015.

<table>
<tr><th>cells number</th><th>fragmentation</th><th>symmetry</th><th>fragmentation</th><th>category</th></tr>
<tr><td>indifferent</td><td colspan="2"></td><td>>50%</td><td rowspan="2">E</td></tr>
<tr><td>0</td><td colspan="3"></td></tr>
<tr><td><6</td><td colspan="3"></td><td rowspan="3">D</td></tr>
<tr><td rowspan="2">≥6 / >12</td><td rowspan="2"></td><td>bad</td><td>indifferent</td></tr>
<tr><td rowspan="2">good</td><td>>20%</td></tr>
<tr><td><20%</td><td rowspan="3">C</td></tr>
<tr><td rowspan="3">[7-12]</td><td>>20</td><td colspan="2"></td></tr>
<tr><td rowspan="2">≤20</td><td>bad</td><td>indifferent</td></tr>
<tr><td rowspan="2">good</td><td>>10-20%</td><td>B</td></tr>
<tr><td>≤10%</td><td>A</td></tr>
</table>

10. STATISTICAL ANALYSIS

In order to evaluate morphological differences among co-cultured and non-co-cultured embryos, the 2-sample test for equality of proportions for frequencies was applied. Briefly, differences were considered statistically significant as long as the p-value was equal or smaller than 0.05. Also, a multifactor ANOVA was performed in order to discover the causes of the embryo development differences between the 21 patients. Here, recipient age, donor age, cigarette smoking, alcohol use, previous pregnancies, prior miscarriages, whether the patient had underwent a cesarean section or a natural birth and infertility causes were the factors studied. Statistical analysis was conducted using the R-commander software (Fox, 2005).

IV. RESULTS AND DISCUSSION

1. ASSESMENT OF MORPHOLOGICAL FEATURES IN CO-CULTURED AND NON-CO-CULTURED EMBRYOS

In this present work, a total number of 297 embryo development cycles were analyzed. Selected embryos belonged to patients ranging in age from 19 to 39 years old. As mentioned hereinbefore, data was obtained from embryos randomly distributed in Geri plates where they were either incubated as co-cultures or non-co-cultures. Specifically, 194 embryos were grown in co-cultured conditions whereas 103 embryos were cultured individually.

- FERTILIZATION RATE

Close scrutiny of the collected data revealed that, for co-cultured embryos, the fertilization rate was higher in comparison with non-co-cultured embryos, as a rate of 85.05% of fertilized embryos with 2 pronucleus (2PN) was obtained for the first group, in marked contrast with the 78.64% percentage observed for the second group. On the other hand, it can be stated that the non-fertilized rate in 0PN embryos was lower in co-cultured embryos than in non-co-cultured. Furthermore, the abnormal fertilization rate was similar in both one pronucleus (1PN) and three pronuclei (3PN) embryos. Finally, a higher rate of degenerated embryos was observed in co-cultured embryos (Table 2).

Table 2. Fertilization rates by ages in co-cultured or non-co-cultures embryos.

Co-culturing	Number of embryos analyzed	Degenerated embryos[1]	0PN[2]	1PN[3]	2PN[4]	3PN[5]
Yes	194	4.64%	8.76%	1.55%	85.05%	0.00%
No	103	2.91%	14.56%	2.91%	78.64%	0.97%

[1] Data given as % of Degenerated embryos / Total number of co-cultured or non-co-cultured embryos.

[2] Data given as % of Non-fertilized embryos / Total number of co-cultured or non-co-cultured embryos.

[3] Data given as % of Abnormal fertilized embryos / Total number of co-cultured or non-co-cultured embryos.

[4] Data given as % of Fertilized embryos / Total number of co-cultured or non-co-cultured embryos.

[5] Data given as % of Abnormal fertilized embryos / Total number of co-cultured or non-co-cultured embryos.

Because of the knowledge obtained from previous work, it was first hypotesized that an increased rate of fertilization in 2PN embryos should be achieved when developing the fertilized embryos as 2 per drop embryos (co-culturing method) instead of 1 per drop (non-co-culture). For this reason, fertilization rates were tested for equality of proportions in order to confirm the mentioned hypothesis. Here, a p-value=0.10 was obtained and so, the alternative hypothesis was refuted since it cannot be confirmed that the differences between fertilization rates are statistically significant. Nevertheless, due to the closeness of the p-value to 0.05 along with the limited sample size, further studies are required using a broader population as these results could fluctuate and our hypothesis be confirmed. Furthermore, considering that fertilization rate values nowadays have been established between 70-80% (Palermo et al., 2009; Neri et al., 2014), the obtained results suggest that co-culturing the embryos could improve their fertilization rate as a percentage of 85.05% was obtained, which is substantially higher than the average.

Regarding the fertilization rates in non-fertilized embryos (OPN), reduced values of non-viable embryos when co-culturing the embryos were expected, as it would confirm that this method is a more reliable source of viable embryos since lower rates of OPN would mean more embryos available for ICSI procedures. Here, the test for equality of proportions was once more applied obtaining a p-value of 0.03, which supports our hypothesis that co-culturing generates a lower number of OPN embryos indeed and so, this technique is a more suitable method to develop them as a lower number of embryos is rejected.

Finally, differences in abnormal and degenerated embryos were assessed. In a similar fashion to OPN embryos, it was expected that fertilization rates in co-cultured embryos would be lower than their non-co-cultured counterparts. Again, a statistical test was applied, obtaining the following p-values; p-value=0.41 for 1PN embryos, p-value=0.16 for 3PN and p-value=0.24for degenerated embryos. Here, significant differences between the two methods cannot be established; nevertheless, the sample since is reduced, so further studies are required to confirm these results.

As previously described in the methodology section, the study resumed considering only those embryos with two pronuclei (2PN) whereas degenerated, non-fertilized (0PN) and abnormal (1PN/3PN) embryos were rejected because of higher risks to give rise to aneuploidies or triploidies. Among co-cultured embryos, those ones who had developed along with degenerated, non-fertilized and abnormal embryos were also discarded as, to date, the interferences that they might cause to the proper development of their counterparts have not been deciphered yet.

Summarizing, the total number of embryos that were used in the upcoming evaluation of the morphological features decreased to 142 for the co-cultured group and to 78 for the non-co-cultured embryos group.

- ## MORPHOLOGICAL EVALUATION AT DAY 3-POST ICSI

As discussed in the methodology of this essay, the three parameters studied were first analyzed independently; number of cells, cellular symmetry and fragmentation degree. Regarding the number of cells, it was expected that at day 3 embryos would have around 7-8 cells as it has been confirmed to be the optimal number of cells to obtain an enhanced rate of implantation (Racowsky et al., 2011). It must be noted that the correlation between number of cells and rate implantation has been a recurrent controversial topic as some studies have stated that embryos possessing >8 or <6 cells at day 3 may degenerate in aneuploid embryos (Magli et al., 2007; Finn et al., 2010).

Nevertheless, in the present study, embryos presenting 7 to 12 cells were considered potential candidates with strong chances of being properly implanted (Figure 3). This assumption was supported by previous evidence showing that the activation of embryonic genome occurs when embryos reach the 8-cell stage and only those with an optimal quality will be able to proceed with their development (Braude et al., 1988). Moreover, lower rates of chromosomal aberrations such as polyploidies are registered (Staessen et al., 2004).

In this case, it was suggested that the co-culturing the embryos might contribute to generate a bigger number of high quality embryos that met the requirements before mentioned. However, when this parameter was compared with non-co-cultured embryos, significant differences were not observed (p-value=0.14).

With regard to the cellular symmetry, the following criteria were established. Briefly, embryos were considered symmetrical when their cells have a similar size. In marked contrast, for those embryos whose cells were not equivalent, they were labeled as non-symmetrical in accordance with the Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology (Figure 3). Significant differences were observed in terms of cellular symmetry when comparing both co-cultured and non-co-cultured embryos, with a p-value of 0.03. Here, the results confirm that the co-culture method generated more symmetrical embryos than the non-co-cultured procedure, achieving a percentage of symmetrical embryos of 90.71% and 80.76%, respectively. These results reaffirm our hypothesis that co-culturing embryos might favor their development as it has been suggested that the lack of cellular symmetry can result in lower rates of implantation (Hnida et al., 2004).

Then, the degree of cellular fragmentation was evaluated. This phenomenon takes place when a cellular fragment without a nucleus is detached from the blastomere (Prados et al., 2012). Briefly, embryos were classified according to their degree of fragmentation into five groups: 0-10%, 10-20%, 20-40%, 40-50% and ≥50% (Figure 3). Again, it was assumed that co-culturing the embryos might prompt some benefits during their first stages of division. Nevertheless, after statistical analysis were applied, significant differences could not be observed between both groups (p-value=0.45). Consequently, it could be said that our hypothesis was erred as co-culturing embryos did not help to avoid cellular fragmentation during their development. Particularly, cellular fragmentation is a key factor for the embryo development since its viability can be critically threatened (Pelinck et al., 2010). Moreover, a close correlation between the fragmentation degree and the number of aneuploidies, as it has been stated that this last increases along with the fragmentation proportionally (Munné et al., 2006). Specifically, low rates of fragmentation (<10%) do not seem to influence the rate implantation (Van Roy-en et al., 2001). In a similar manner, embryos not exceeding rates of 20%-25% of fragmentation are not compromised, but greater ones could be (Ziebe et al., 1997; Racowsky et al., 2003).

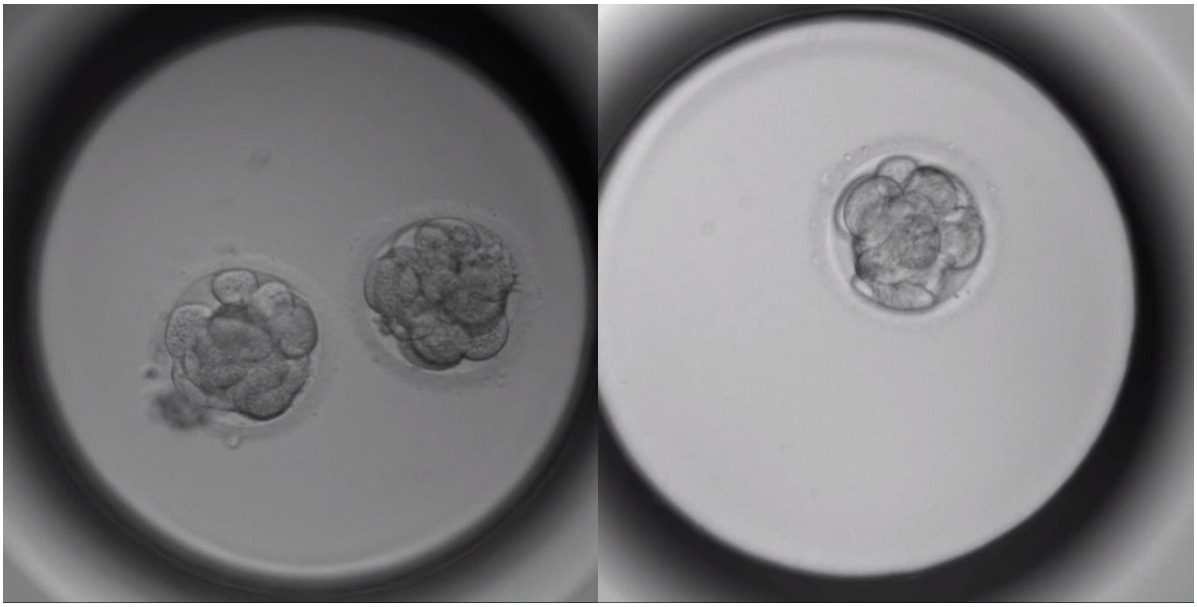

Figure 3. Embryos photographs obtained by means of the time-lapse incubator of co-cultured embryos (left) and non-co-cultured embryos (right) at day 3. Self-source.

Finally, once those parameters were defined individually, embryos were again classified combining these same factors: number of cells, cellular symmetry and cellular fragmentation. To this end, the classification detailed at the methodology section was used. Here, it was expected to obtain high percentages of type A and B for co-cultured embryos, but low percentages for C, D and E in this same group. Nevertheless, data showed similar frequencies among co-cultured and non-co-cultured embryos (Table 3), and no significant differences were confirmed in the statistics analyses, with a p-value = 0.44 for A embryos, p-value = 0.42 for B embryos, p-value = 0.59 for C embryos, p-value = 0.32 for D embryos, and p-value = 0.60 for E embryos.

Table 3. Morphological classification rates on day 3 for co-cultured and non-co-cultured embryos.

Co-culturing	Number of Fertilized Embryos	D3 A[1]	D3B[2]	D3C[3]	D3D[4]	D3E[5]
Yes	142	54.23%	7.04%	26.06%	8.45%	4.23%
No	78	58.97%	8.97%	14.10%	15.38%	2.56%

[1] Data given as % of A embryos / Total number of co-cultured or non-co-cultured fertilized embryos.

[2] Data given as % of B embryos / Total number of co-cultured or non-co-cultured fertilized embryos.

[3] Data given as % of C embryos / Total number of co-cultured or non-co-cultured fertilized embryos.

[4] Data given as % of D embryos / Total number of co-cultured or non-co-cultured fertilized embryos.

[5] Data given as % of E embryos / Total number of co-cultured or non-co-cultured fertilized embryos.

- ## COMPACTION STAGE AT DAY 4-POST ICSI

Another aspect evaluated included in the morphological analysis was the compaction stage if the embryos. Specifically, two groups were defined according to their degree of compaction; compacted embryos and non-compacted embryos (Figure 4). Here, a total of 76.76% compacted embryos was obtained in the co-cultured group, while only a 69.23% of embryos were on the compacted stage when the non-co-cultured method was applied. Despite the existing differences, they were not statistically significant when compared (p-value=0.11).

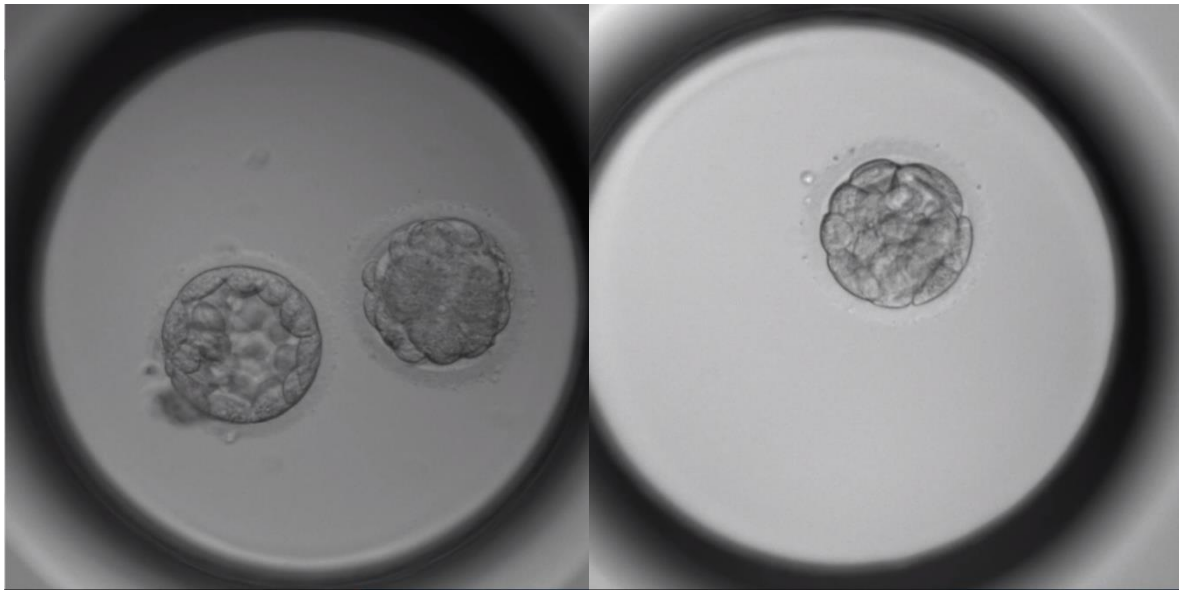

Figure 4. Embryos photographs obtained by means of the time-lapse incubator of co-cultured embryos (left) and non-co-cultured embryos (right) at day 4. Self-source.

To obtain more compacted embryos would be extremely favorable as the compaction is an essential requirement for the genomic activation and so, for the proper embryonic development (Behr et al., 2000; Alikani. 2005). Despite being a key factor for the developing phase, scarce research has been conducted in order to elucidate which is the influence of the compaction stage at day 4 to the implantation rate (ASEBIR, 2015; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011). Furthermore, the degree of compaction is another critical requirement as those embryos compacted in less than a 50% will have a lower predisposition to implantation (Tao et al., 2002).

- ## % BLASTOCYST AT DAY 5, DAY 6, AND DAY 7

In the following section, the main goal was to determine the frequencies of embryos achieving the blastocyst stage at day 5, day 6 and day 7 respectively along with the percentages of discarded embryos (Table 4).

Table 4. Blastocyst stage embryos frequencies at day 5, day 6 and day 7.

Co-culturing	Number of Fertilized Embryos	Blastocyst D5[1]	Blastocyst D6[2]	Blastocyst D7[3]	Discarded Embryos[4]
Yes	142	58.45%	19.01%	0.00%	22.54%
No	78	46.15%	19.23%	1.28%	33.33%

[1] Data given as % of Number of blastocysts at day 5 / Total number of co-cultured or non-co-cultured fertilized embryos.

[2] Data given as % of Number of blastocysts at day 6 / Total number of co-cultured or non-co-cultured fertilized embryos.

[3] Data given as % of Number of blastocysts at day 7 / Total number of co-cultured or non-co-cultured fertilized embryos.

[4] Data given as % of Number of discarded embryos / Total number of co-cultured or non-co-cultured fertilized embryos.

It was suggested that at day 5, higher rates of blastocysts should be reached when using the co-culture method which would mean that this strategy truly improves the alternative methods available to date. Here, a total of 58.45% of the initially fertilized embryos reached the blastocyst stage of the co-cultured embryos group, while the percentage of blastocysts for non-co-cultured embryos was only of 46.15%. This values fit in the current interval of embryos reaching the blastocyst stages, which ranges from 40-60% (Gardner et al.,1999). However, it must be noted that the frequency of blastocyst in the 2 per drop group is extremely close to the upper limit whether for non-co-cultured embryos is closer to the lower limit. Furthermore, differences between co-cultured and non-co-cultured groups were statistically significant ($p=0.04953$), confirming the superiority of our strategy. This increment of embryos reaching the blastocyst stage at day should lead to a decrease of the number of those achieving this state at day 6 and 7 and/or the number of discarded.

In the last decades, several studies have evidenced that embryos reaching the blastocyst stage at day 6 or day 7 are considered of lower quality and less susceptible to implant effectively as compared with embryos maturing at day 5 since they develop slower than their counterparts (Shapiro et al., 2001; Kovalevsky et al., 2013). However, others have evidenced their potential of implantation as long as they meet some quality requirements. For this reason, day 6- and day 7-blastocysts were also taken into account in this study. As discussed before, reduced frequencies of blastocysts occurring at day 6 and 7 were

expected when evaluating embryos derived from co-cultures which would entail that blastocyst with an enhanced quality were generated when using this method. Although our assumption was supported with previous data, comparison of the frequencies of co-cultured and non-co-cultured revealed that they were not significantly different, and at day 6 (p=0.9305) and day 7 (p=0.1763). Summarizing, co-culturing embryos do not reduce the number of blastocysts at day 6 or 7 but neither do rise.

Regarding the discarded embryos, the co-cultured group presented lower rates than the non-co-cultured group, being this difference statistically significant (p=0.04102) and so, confirming that the number of discarded embryos using this strategy is reduced. Therefore, with the present results obtained, it can be stated that co-culturing supports the embryo development by promoting a higher generation of blastocysts and reducing the number of discarded embryos.

- ## EVALUATION OF BLASTOCYST QUALITY

Subsequently, a morphology evaluation of the blastocysts was performed. Embryos were classified according to Gardner system (Gardner et al., 1998). Which consists in a three-part scoring system, where the first character refers to the blastocel expansion, the second to the inner cell mass (ICM), and the last character to the trophectoderm. Moreover, in order to specify their stage of blastocoel expansion, a numeric system is used to allocate them where 3 refers to no expansion, 4 is for expanded blastocysts, 5 refers to hatching embryos and 6 is used for hatched embryos. Regarding the inner cell mass (ICM), A corresponds to a compact ICM, B to non-compact, C when the ICM>1900 μm^2, D when signs of degeneration are appreciated, and E when is degenerated. Again, the trophectoderm is also classified using capital letters. Briefly, A refers to an homogeneous trophectoderm, cohesive and with a great number of cells, B refers to a homogeneous trophectoderm with a low number of cells, C when presents a little amount of cells, D when signs of degeneration are observed and D for degenerated trophectoderm (ASEBIR, 2015).

At day 5, the highest quality blastocysts were defined as 4-5AA/AB/BA/BB, which were confirmed as the most suitable blastocysts to transfer (Figure 5). The proportion of best blastocysts in co-cultured embryos was 50.70% and in 43.59% in non-co-cultured embryos, but these values were not statistically significant although the p-value was almost under 0.05 (p=0.07). So, it can be said that differences in terms of blastocyst quality among the different embryos could not be discerned.

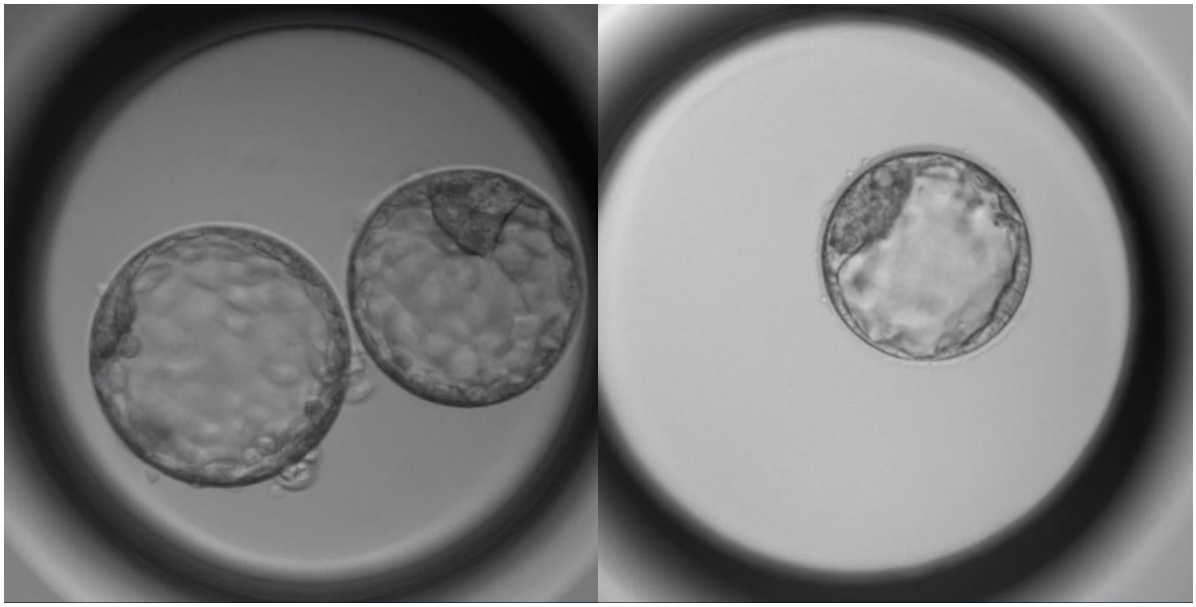

Figure 5. Blastocysts photographs obtained by means of the time-lapse incubator of co-cultured embryos (left) and non-co-cultured embryos (right) at day 5. Self-source.

In order to choose the best candidate to perform the embryo transference, it is very important to possess helpful tools that facilitate the researcher's task of classifying them and so, the most suitable blastocyst is selected. Thus, a great number of studies have been elaborated with the same goal to create a consensus for the classification of the blastocysts. According to Gardner et al. (2000), the morphological evaluation of the trofectoderm and the ICM enables to accurately predict the rate of implantation. Particularly, the evaluation of the trophectoderm in recent years has effectively enabled to predict the rate of implantation (Ahlström et al, 2011; Hill et al., 2013). Moreover, the study of the trophectoderm is also useful since it has been confirmed that compromised morphologies might lead to more aneuploidies (Alfarawati S, 2011). In discordance, others approaches have emphasized that the ICM should be more important when classifying the embryos as it has been observed that those embryos with a bigger ICM will be more prompt to be implanted (Richter et al., 2001). Finally, the blastocoel expansion is also an important predictor as the most expanded embryos will be more viable and their rate of implantation will be higher (Racowsky et al., 2003).

- ## CRYOPRESERVATION AND TRANSFERENCE OF EMBRYOS

Those blastocysts selected through Gardner system and that in this way, considered the best quality embryos, were cryopreserved. From the 142 fertilized embryos performing the co-culturing, a 68.18% were cryopreserved, and a 31.82% of embryos were cryopreserved in the non-co-cultured embryos group. It is noticed that a greater number of co-cultured embryos were cryopreserved in contrast with non-co-cultivated embryos, indicating a higher amount of suitable embryos to be transferred with a better quality in the co-cultured embryos group. The differences in the number of cryopreserved embryos were evaluated in order to determine whether or not these differences are because of the co-culturing. Although p-value was almost smaller than 0.05 (p=0.10), the present differences weren't significantly explained by the co-culturing factor.

Concerning the transferred blastocysts rates, these were obtained by performing a ratio among the transferred embryos from co-cultured or non-co-cultured group and the total embryos transferred. Selected blastocysts for transference are those of the best quality embryos per patient. Thus, higher transferred blastocysts rates from co-cultured embryos group would state better quality blastocysts in this embryos group. A 72.22% of transferred blastocysts come from the co-cultured group, and a 27.78% were transferred from non-co-cultured group. Those differences were significantly explained by the co-culturing factor (p-value = 0.04). Hence, the co-culturing affects the embryo transfer.

A great number of studies have established the imputs of blastocyst transfer. According to Gardner et al. (2000, 2004) the blastocyst transfer allow a better embryonary selection, as well as a better synchrony among the embryo and the endometrium (Milki et al., 2000; Blake et al., 2007) and a lower uterine contractibility during the embryo transfer process (Fanchin et al., 2001). The extended culture permits the best embryo detection and a reduction of the chromosomal abnormalities during the embryo development that increases the likelihood of an evolutive pregnancy (Papanikolaou et al., 2005). In addition, this method possesses higher implantation rates (Papanikolau et al., 2004).

In the other hand, former studies claimed no improvements in the blastocysts transfer in contrast with day 3 embryo transfer (Bungum et al., 2003; Blanke et al 2004). Nevertheless, further studies confirmed that embryo transfer performed at other time points using cleavage-stage embryos at day 3 results in lower implantation rates (Papanikolau et al., 2004; Scholtes et al., 2006). Hence, for the development of this study, transfer method was performed using blastocysts due to the great amount of studies proposing as a better method the blastocyst transfer.

- % IMPLANTATION RATE

In order to determine whether or not the co-culturing method had an influence in the implantation rate, two parameters directly correlated with the implantation were also evaluated along with the clinical implantation; the hCG blood test and the evolutive implantation. For all the parameters, it was expected that better implantation rates derived from co-cultured embryos. In reference to the hCG blood test, a 73.33% of co-cultured embryos had a positive test while in non-co-cultured a 42.86% of embryos had a positive test. On the other hand, evolutive implantation rates in the co-cultured group were 60%, in marked contrast with the non-co-cultured group which remained 28.57%. Finally, implantation rates were calculated from the ratio among the number of implanted embryos from co-cultured or non-co-cultured groups and the total number of implanted embryos. In the co-cultured group, a 66.67% of embryos lead to clinical implantation, whereas in the non-co-cultured group, clinical implantation was reached for 28.57% of embryos. Statistical analysis revealed that differences between methods for both “indirect” analysis, the hCG blood test and evolutive implantation, were not statistically significant (p-value=0.08 and p=0.08 respectively). In high contrast, for the clinical implantation, the p-value obtained was 0.04, confirming that in overall terms, co-culture of embryos is an efficient method to improve the implantation rate in comparison with the non-culturing strategies.

These results are highly encouraging as the final goal of every IVF treatment is to maximize the rate of implantation and consequently, gestation and birth rates. For this reason, efforts should be focused in developing systems such as the co-culturing method in order to increase the predisposition of the embryos to be implanted and their quality. Therefore, better rates of implantation might enable to implant a single embryo and so, reduce the number of multiple gestations. However, this breakthrough will only be possible if the most viable embryo can be selected (Gardner et al., 1998; Gardner et al., 2000), and the transference of the embryo is performed at the blastocyst stage (Vidaeff et al., 2000; Thompson et al., 2013).

2. PATIENT’S AGE EFFECT IN THE MORPHOLOGICAL EMBRYO DEVELOPMENT

To analyze the influence of the patient’s age in co-cultured and non-co-cultured embryos, an age threshold was established as mentioned hereinbefore. In the co-cultured group, the total number of studied embryos in patients under 35 years old (<35) was 124, and for patients equal or greater than 35 years old (≥35) the number of embryos was 70. For the non-co-

cultured group, 68 embryos from <35 years old patients and 35 embryos from ≥35 years old patients were evaluated. Again, the same parameters described in the previous section were studied, but this time the age threshold was also considered.

For all parameters evaluated, the expected results were that in both <35 years old patients and ≥35 years old patients age, co-culturing would allow to improve their frequencies. In order to confirm that hypothesis, the following analyses were performed.

The obtained data for fertilization rate revealed that in <35 years old patients the differences in fertilization (p=0.4591), non-fertilized embryos (p=0.1488), abnormal fertilizations (p=0.8534) and degenerated embryos (p=0.1289) frequencies among both co-cultured and non-co-cultured embryos were not statistically significant, , , and. Therefore, the patient's age factor had no effect in the co-cultured and non-co-cultured embryo fertilization. On the other hand, for ≥35 years old patients, the fertilization rate in co-cultured embryos was higher than non-co-cultured embryos, as a rate of 85.71% was obtained for the first group, and a rate of 71.43% for the second group, despite such differences were not significant (p=0.0741). Consequently, it can be said that the present differences among co-cultured and non-co-cultured embryos were not originated by the patient's age factor. Nevertheless, as in mentioned before, due to the closeness of the p-value to 0.05 along with the limited sample size, further studies are required using a broader population. For the non-fertilized embryos, abnormal fertilizations and degenerated embryos no significant differences appeared due to the respective p-values were above 0.05 (p-value=0.12, p-value=0.13, p-value=0.87, respectively).

Regarding the morphological evaluation at day 3-post ICSI, for the number of cells significant differences were not observed nor in <35 years old patients (p-value=0.08) nor ≥35 years old patients (p-value=0.17), thus the patient's age factor has no effect in the cleavage process. With regard to the cellular symmetry, no significant differences were observed in <35 years old patients (p-value=0.47) while significant differences were obtained in ≥35 years old patients (p-value=0.04) between co-cultured and non-co-cultured embryos. Here, results confirm that in older patients, the co-cultured method generated more symmetrical embryos when compared with the non-co-cultured, achieving percentages of 95.65% and 71.43% of symmetrical embryos respectively. So, the co-culturing provides benefits in the embryo development for ≥35 years old patients. Then, the degree of cellular fragmentation was evaluated. No significant differences were observed between <35 years old patients (p-value=0.95) nor ≥35 years old patients (p-value=0.34) in both co-cultured and non-co-cultured embryos.

Regarding the day 3 embryo classification, it was expected to obtain higher rates of type A and B in co-cultured embryos but low percentages for C, D and E in this same group. Nevertheless, data showed similar frequencies among co-cultured and non-co-cultured embryos for both groups of patient's age, and no significant differences were confirmed in the statistics analyses, with a p-value=0.32 for A embryos, p-value=0.58 for B embryos, p-value=0.35 for D embryos, and p-value=0.82 for E embryos in <35 years old patients, and a p-value=0.77 for A embryos, p-value=0.87 for B embryos, p-value=0.77 for C embryos, p-value=0.13 for D embryos, and p-value=0.34 for E embryos in ≥35 years old patients. The only parameter that showed significant differences was the C classified embryos, with a p-value = 0.03.

The evaluation of the compaction stage showed no significant differences between the co-cultured and non-co-cultured embryos for both <35 years old patients (p-value=0.34) and ≥35 years old patients (p-value=0.48). So, it could be said that the patient's age has no effect in the compaction stage.

Regarding the analysis of blastocysts at day 5, day 6 and day 7 and the discarded embryos, similar frequencies were obtained in the <35 years old patients evaluation in both co-cultured and non-co-cultured embryos, confirmed by the respective p-values (p=0.54, p=0.60, p=0.20, p=0.38). In the ≥35 year's old patients, higher rates were obtained in co-cultured embryos, as a total of 67.31% of the initially fertilized embryos reached the blastocyst stage of co-cultured embryos, while the percentage of blastocysts for non-co-cultured embryos was only of 40.00%; however, no significant differences were found (p=0.06). Further studies are required using a broader population as these results could fluctuate and our hypothesis be confirmed. Blastocysts at day 6 (p=0.57) and discarded embryos (p=0.09) showed no significant differences as well. In the ≥35 year's old patients, there were no blastocysts at day 7. Hence, the patient's age does not explain the differences between the co-cultured and non-co-cultured embryos.

Neither in the morphology evaluation of the blastocysts, no significant differences were found in both <35 years old patients (p-value=0.79) and ≥35 years old patients (p-value=0.41). Thus, the patient's age doesn't improve the blastocysts quality for co-cultured and non-co-cultured embryos.

As regards of the cryopreserved embryos, no significant differences were found between the co-cultured and non-co-cultured embryos in both <35 years old patients (p-value=0.35) and ≥35 years old patients (p-value=0.34), confirming once again no effect of the patient's age in the co-cultured and non-co-cultured embryos.

For the implantation rate analysis, no significant differences were found between the co-cultured and non-co-cultured embryos <35 years old patients for the hCG blood test (p-value=0.15), and the evolutive implantation (p-value=0.13). The only parameter that showed significant differences the clinical implantation (p-value=0.04). Thus, in <35 years old patients, the co-culturing improves the clinical implantation. For the ≥35 years old patients all blastocysts implanted were from the co-cultured embryo group.

V. CONCLUSIONS

The main conclusions of this work are summarized below:

1. Embryo co-culturing increases the percentage of blastocysts at day 5 of embryo development, as well as it minimizes the number of discarded embryos that do not reach the blastocyst stage. In addition, the number of transferred blastocyst is higher for co-cultured embryos, proving a better blastocysts quality. Finally, it should be pointed a higher clinical implantation.
2. Embryo co-culturing may improve fertilization rate, as well as some parameters studied in the late stage of embryo development, as an embryo quality improvement and an increase of the cryopreserved embryos.
3. Strengths of our study include that embryos were randomly distributed into co-cultured and non-co-cultured plate, and its treatment was carried out following the laboratory consensus established by using breakthrough technology.
4. Limitations to this study include the low sample size of embryos that could have triggered a loss of the study strength. For the upcoming studies, this can be solved by using a broader population. In addition, a prior embryo selection was carried out, excluding those patients with incomplete embryo development and a poor genetic prognosis.
5. The majority of the data was obtained from <35 years old patients, that usually present better blastocyst quality and higher implantation rates. Nonetheless, after the application threshold age, differences among co-cultured and non-co-cultured embryos in <35 years old and ≥35 years old patients were established, where co-culturing has a bigger effect in ≥35 years old patient improving the clinical implantation than the <35 years old patients. Thus, a specialized study can be proposed to determine the effects of co-culturing in patients with a higher age.
6. Finally, due to the encouraging results obtained in the current study, more studies have to be done in the embryo co-culturing field that can lead to an emerging culturing method.

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